A prospective birth cohort study on cord blood folate subtypes and risk of autism spectrum disorder

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ABSTRACT

Background: We previously reported that extremely high concentrations of maternal plasma folate were associated with increased risk of autism spectrum disorder (ASD) in children. This study explored whether specific types of folate in cord blood have differential association with ASD.

Objectives: In the Boston Birth Cohort (BBC), we assessed the association between cord blood unmetabolized folic acid (UMFA), 5-methyl tetrahydrofolate (THF), and total folate and a child's ASD risk. In a subset, we explored whether the association between UMFA and ASD risk can be affected by the dihydrofolate reductase (*DHFR*) genotype and cord plasma creatinine. We also examined prenatal correlates of cord UMFA concentrations.

Methods: This report included 567 BBC children (92 ASD, 475 neurotypical), who were recruited at birth and prospectively followed at the Boston Medical Center. ASD was defined from International Classification of Diseases (ICD)-9 and ICD-10 codes documented in electronic medical records.

Results: Children with cord UMFA in the highest, versus lowest quartile, had a greater ASD risk (adjusted OR, $aOR_{quartile4}$: 2.26; 95% CI: 1.08, 4.75). When stratified by race/ethnicity, the association was limited to 311 (45 ASD) Black children ($aOR_{quartile4}$: 9.85; 95% CI: 2.53, 38.31); a test of interaction between race/ethnicity and cord UMFA concentrations was significant ($P = 0.007$). The UMFA-ASD association in Black children slightly attenuated after adjusting for cord plasma creatinine ($P = 0.05$). There was no significant association between cord 5-methyl THF, total folate, *DHFR* genotype, and ASD risk. Cord total folate and maternal supplement intake during second trimester were associated with higher cord UMFA.

Conclusions: Higher concentrations of cord UMFA, but not 5-methyl THF or total folate, were associated with a greater risk of ASD in Black children. This study in a preterm-birth-enriched cohort raises more questions than it could answer and underscores the need for additional investigations on the sources and role of cord UMFA in children's neurodevelopmental outcomes and underlying mechanisms. *Am J Clin Nutr* 2020;112:1304–1317.

Keywords: folate, folic acid, unmetabolized folic acid, autism, ASD, pregnancy

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by impairments in communication and presence of restrictive, repetitive behaviors and interests (1). According to the latest CDC estimates, 1 in 54 children who are

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Data described in the manuscript, code book, and analytic code will be made available upon request pending Institutional Review Board review and approval.

Supplemental Tables 1–10 and Supplemental Figures 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BBC, Boston Birth Cohort; DHFR, dihydrofolate reductase; EMR, electronic medical records; ICD, International Classification of Diseases; ID, intellectual disabilities; THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

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aged 8 y in the USA have ASD (2). The etiology of ASD is still elusive; although it is highly heritable, there is some consensus that ASD may also have environmental underpinnings and likely prenatal origins (1). Emerging evidence suggests that ASD might be a systemic abnormality involving multiorgan systems and metabolism, with the folate-methionine cycle playing an important role in the ASD etiology (3–5).

Folates are an integral part of 1-carbon metabolism and are involved in many important biological functions including DNA synthesis and methylation (6). Adequate maternal folate status is important to reduce the risk of neural tube defects (7). Folates are essential B vitamins because humans are incapable of folate synthesis, and thus require dietary intake. Dietary folate is comprised of natural folates (found in foods) and folic acid (a synthetic fully oxidized form added to fortified foods and supplements). Folic acid is biologically inert and requires dihydrofolate reductase (*DHFR*) for its conversion to tetrahydrofolate (THF), after which it is subsequently integrated into the body's folate pool (8, 9). When the intake of folic acid exceeds the metabolic capacity of the body, unmetabolized folic acid (UMFA) appears in the plasma. Studies have shown that the 19-bp deletion allele in the *DHFR* gene is associated with higher circulating folic acid, especially with high folic acid intake (8, 9).

Many epidemiologic studies, have shown that prenatal folic acid intake (primarily based on self-reported supplement intake) may be protective against ASD (10–16). However, a few other studies have not confirmed these findings (17, 18). Even in studies that reported an association with prenatal supplement intake, associations between maternal folate biomarkers and ASD were inconsistent, particularly around conception and pregnancy timing (10, 15, 19). The basis for these discrepant findings is unclear but likely attributable to timing, dosage, baseline folate status, genetic polymorphism, residual confounding, and several other factors (8, 10, 11, 15–17, 20, 21).

The present study extends our previous report that among mother-infant pairs enrolled in the Boston Birth Cohort (BBC), extreme low and high concentrations of maternal plasma folate 2–3 d after birth (a proxy of maternal 3rd trimester folate concentration) were associated with an increased risk of ASD, suggesting a U-shaped risk curve (22). Here, we investigated whether different folate forms (total folate, 5-methyl THF, and UMFA) in cord blood at birth (reflecting fetal exposure in utero) were associated with a differential risk of a child's ASD. In a subset, we further assessed whether cord creatinine, an indicator of kidney function, influenced the association between cord UMFA and ASD. We also explored which prenatal factors may influence cord blood UMFA concentrations, including maternal folate concentrations, a child's *DHFR* 19-bp del polymorphism, and maternal race/ethnicity.

Methods

Participation and data collection procedures

The BBC is a prospective cohort that was initiated in 1998 to study the environmental and genetic determinants of preterm birth and early life origins of pediatric and chronic diseases. Between 1998 and 2014, mothers who delivered a singleton live birth at the Boston Medical Center were invited to participate in the study. Those with multiple-gestation pregnancies, chromosomal abnormalities, major birth defects, and preterm deliveries as a result of maternal trauma were excluded. More than 90% of the mothers who were approached agreed to participate and were enrolled into the study (23, 24). This cohort oversampled mothers who had preterm infants such that for every preterm (defined as <37 weeks of gestation) and/or low birth weight infant (defined as <2500 g), about 2 term and normal weight infants (and their mothers) were enrolled in the study (23, 24). The BBC is thus a preterm-enriched cohort; and preterm birth is a known risk factor for ASD.

After obtaining informed consent, mothers were interviewed 24–72 h after delivery using a standardized questionnaire to collect demographic, medical, reproductive history, nutrition, and substance use data. In addition, a standardized abstraction form was used to gather data from a medical records review, including prenatal and intrapartum clinical care, pregnancy complications, birth outcomes, and laboratory test results. A subset of the BBC children who continued to receive pediatric care at the Boston Medical Center were followed-up from birth to ≤21 y and were included in this report. As reported previously, there were no major differences in baseline demographic and clinical characteristics between those that continued to be part of the postnatal follow-up cohort and those that did not (25). Electronic medical records (EMR) with clinicians' primary and secondary diagnoses using International Classification of Diseases (ICD)-9 and ICD-10 codes were obtained for every postnatal clinical visit since 2003. The Institutional Review Boards of the Johns Hopkins Bloomberg School of Public Health and Boston Medical Center approved this study.

Exposure

Umbilical cord blood samples were collected at the time of delivery. The blood samples were separated into plasma and white blood cells and RBCs and were stored in a freezer at −80°C until the analysis for total folate, 5-methyl THF, and UMFA.

Cord blood UMFA was measured using the LC-MS/MS method (26). Using an AB SCIEX QTRAP 5500 system from Applied Biosystems, deproteinized plasma samples were analyzed for UMFA at the laboratory of Dr. Jacob Selhub at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University (27). UMFA was quantified and identified by comparing with external and internal standards of known concentration. The limit of detection for folic acid and 5-methyl THF was 0.1 nmol/L and 0.08 nmol/L, respectively. For each sample, the plasma concentration of total folate was calculated as a sum of UMFA and 5-methyl THF. In order to assess the laboratory precision, blinded replicate quality control plasma samples were randomly interspersed among the participant samples. Interassay CVs were 6.28% for cord folic acid and 9.47% for 5-methyl THF. Laboratory personnel had no knowledge of the neurodevelopmental outcomes of the study children.

Creatinine in the cord blood was assessed as part of a metabolome panel and was measured using the LC-MS/MS technique (28). The *DHFR* 19-bp polymorphism (rs70991108) genotypes for the infants were determined with the use of Taqman allelic discrimination (9). Genotyping assay accuracy was verified by repeating 3 random study samples and also

by genotyping 3 control samples that had been previously genotypically verified by sequencing.

Outcome

Each child's neurodevelopmental outcomes were obtained from their EMR. Children born by 2014 that were ever diagnosed with autism (ICD-9 code 299.00, 299.01; ICD-10 code 84.0), Asperger syndrome (ICD-9 code 299.80; ICD-10 code 84.5, 84.8), and/or pervasive developmental disorder not otherwise specified (ICD-9 code 299.90; ICD-10 code 84.9) were categorized as having ASD. Neurotypical children were those that did not have ASD, attention deficit hyperactivity disorder (ADHD), intellectual disabilities (ID), and other developmental disabilities. Those children who had concurrent diagnosis such as ASD and ADHD (ICD-9 code 314.01), or ASD and ID (ICD-9 code 319), were still categorized as having ASD.

Covariates

The covariates that were adjusted in the analysis include: maternal age at delivery, smoking during pregnancy (ever smoked 3 mo before pregnancy/during pregnancy versus not smoked before pregnancy/during pregnancy), parity (not including the index pregnancy), maternal education (high school or less versus some college or more), maternal prepregnancy BMI, maternal diabetes status (no pre-existing diabetes versus pre-existing diabetes mellitus versus gestational diabetes mellitus), child's sex (female versus male), gestational age at birth (term, defined as ≥37 completed weeks of gestation versus preterm, defined as <37 weeks of gestation), year of the infant's birth (continuous variable), and self-reported race/ethnicity (Black, white, Hispanic, and other). Whites and Hispanics included all individuals that identified themselves as whites and Hispanics, respectively; Blacks included self-reported Black, African Americans, Haitian, and Cape Verdeans race and ethnicities; and other included Asian and Pacific Islanders, and individuals with a mixed or other racial background (29).

Statistical analysis

Folate forms (UMFA, 5-methyl THF, and total folate) in cord blood were considered as exposures for association with ASD versus neurotypical status. Data analyses were performed to compare the characteristics of neurotypical children and those with ASD using the chi-square test for categorical variables and ANOVA for continuous variables. Predetermined logistic regression models were applied to assess the crude and adjusted associations between cord total folate, 5-methyl THF, UMFA concentrations (independent variables), and ASD status (dependent variable), which was defined as a binary variable with neurotypical children as the reference group. Covariates were selected a priori based on existing literature, including our own work in the BBC (22, 25, 30–32). Our final model adjusted for maternal age, maternal smoking during pregnancy, parity, maternal education, maternal prepregnancy BMI, maternal diabetes status, race/ethnicity, child's sex, gestational age at birth, and year of the infant's birth. Potential effect modification of the association between UMFA and ASD by race/ethnicity, preterm birth status, and infant sex was evaluated by including appropriate interaction terms in the logistic regression model previously mentioned.

Sensitivity analysis was conducted to assess whether EMR misclassification of ASD or neurotypical development influenced study findings, by applying stringent criteria for ASD diagnosis (defined as ASD code for ≥2 visits, including a visit to a developmental specialist). Potential effect modification by other important covariables were explored in subgroup analyses. Additional subgroup analyses were conducted in a subset with available cord creatinine data to assess whether kidney function influenced the association between UMFA and ASD. All results are presented as ORs (95% CI). Two-sided tests were used with a 0.05 significance level. Data were analyzed using STATA version 13.0 (StataCorp).

Results

A total of 567 children who had folate species data measured in cord blood were included in the analysis, of which 92 were ASD cases and 475 were children with neurotypical development (**Figure 1** and **Supplementary Figure 1**). Of the 92 children with ASD, 33 had co-occurring ADHD, 9 had ID, and 88 had other developmental disabilities. A summary of demographic and clinical characteristics of mothers and children is presented in **Table 1** and more details have been previously published (25, 29, 31). We found that maternal characteristics such as higher maternal age and BMI, and child characteristics such as male sex, preterm birth, and year of birth (born after 2007) were more frequently observed in ASD children in this sample. Maternal conditions such as hemolysis, elevated liver enzymes, low platelet count syndrome (HELLP), pre-eclampsia, and epilepsy were not significantly different by a child's ASD status. The frequency of maternal supplement intake during preconception and pregnancy (1st, 2nd, and 3rd trimesters) also did not differ by ASD status.

Figure 2 shows the distribution of log-transformed cord UMFA, 5-methyl THF, and total folate concentrations among neurotypical and ASD children for the entire sample (top panel) and restricted to Black children (bottom panel). Although the distributions overlapped for the most part, there was a right-shift in the distribution of cord UMFA for Black children with ASD (P <0.05) (Figure 2, panel D). The distribution for Hispanics is presented in **Supplementary Figure 2**.

The median cord UMFA concentration was 0.69 nmol/L (IQR: 0.36–1.33 nmol/L). The mean cord UMFA did not differ significantly between neurotypical and ASD children, nor did mean total folate and 5-methyl THF. However, mean cord UMFA differed by race with Black children having significantly higher UMFA than non-Black children (1.44 nmol/L [SD = 3.30] versus 0.98 nmol/L [SD = 1.04]). There was a very strong correlation between cord total folate and cord 5-methyl THF (r = 0.99, P <0.001), but moderate correlations between cord UMFA and cord total folate (r = 0.39, P <0.001) and cord 5-methyl THF (r = 0.36, P <0.001). The correlation between maternal blood folate (n = 436) and folate species (total folate [r = 0.12, P = 0.01], 5-methyl THF [r = 0.12, P = 0.001], and UMFA [r = 0.16, P <0.001]) was modest (**Table 2**).

We assessed the prospective association between log-transformed cord UMFA and subsequent ASD risk and found that

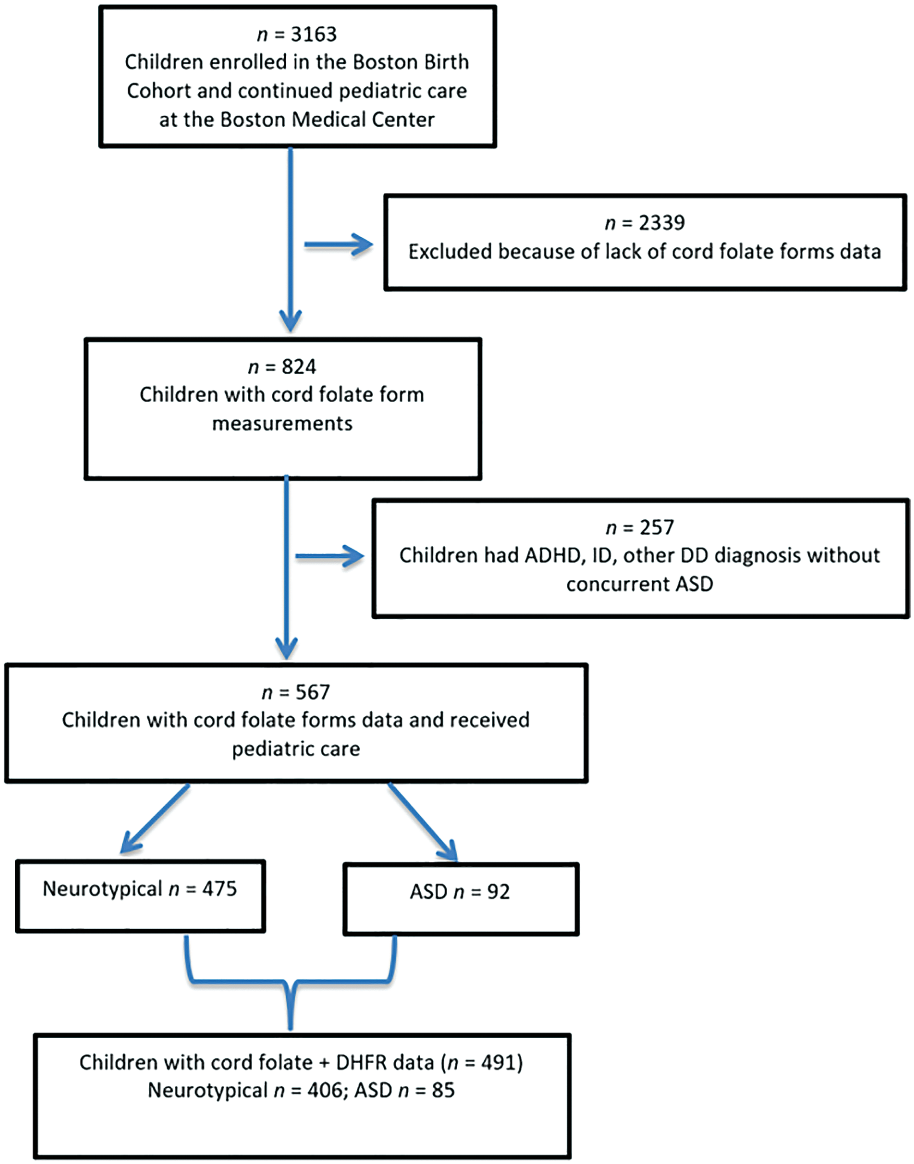

FIGURE 1 Flow chart of study sample inclusion and exclusions. ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BMC, Boston Medical Center; DD, developmental disabilities; DHFR, dihydrofolate reductase; ID, intellectual disabilities.

children with cord UMFA in the highest quartile, compared with those in the lowest quartile, had a greater ASD risk ($OR_{quartile4}$: 2.37; 95% CI: 1.19, 4.70) (**Table 3**). This association remained significant after adjusting for covariates specified in model 1 including maternal age, education, parity, smoking status, diabetes, BMI, race/ethnicity, child's sex, preterm status, and year of birth ($aOR_{quartile4}$: 2.26; 95% CI: 1.08, 4.75). Further adjusting for cord 5-methyl THF (model 2) slightly attenuated the association ($aOR_{quartile4}$: 2.14; 95% CI: 0.98, 4.65), but the results were consistent after adjusting for *DHFR* genotype in addition to 5-methyl THF ($aOR_{quartile4}$: 2.50; 95% CI: 1.05, 5.92) (model 3).

In the subgroup analyses limited only to Black children, there was a dose-response relation between increased cord UMFA concentrations and ASD risk. Compared with Black children who had the lowest cord UMFA (quartile 1), those with UMFA in all the other quartiles had increased ASD risk ($aOR_{quartile2}$: 5.06; 95% CI: 1.19, 21.56; $aOR_{quartile3}$: 5.99; 95% CI: 1.46, 24.66; $aOR_{quartile4}$: 9.85; 95% CI: 2.53, 38.31). There was a statistically significant interaction between race/ethnicity and cord UMFA concentrations in crude and adjusted models ($P = 0.007$). The association between cord UMFA and ASD in non-Black children was not statistically significant in both crude ($OR_{quartile4}$: 1.00; 95% CI: 0.41, 2.43) and adjusted models ($OR_{quartile4}$: 0.85; 95% CI: 0.30, 2.40) (**Table 3**).

We repeated the main analyses by restricting the sample to other high-risk subgroups (i.e., boys, children born preterm). Among boys, the highest cord UMFA was significantly associated with increased ASD risk in the adjusted model ($aOR_{quartile4}$: 3.10; 95% CI: 1.22, 7.88). However, the interaction between sex and cord UMFA was not statistically significant ($P > 0.05$). Among Black boys, there was a statistically significant

TABLE 1 Maternal and child characteristics by child ASD status

Characteristics	Neurotypical ($n = 475$)	ASD ($n = 92$)	P value
Mother			
Age at birth, y (mean ± SD)	28.3 ± 6.4	29.8 ± 6.2	0.04
Parity (%)			0.90
0	210 (44.2)	40 (43.5)	
1 or more	265 (55.8)	52 (56.5)	
Mother's education (%)			0.09
High school or less	303 (63.8)	49 (53.3)	
Some college or more	168 (35.4)	43 (46.7)	
Missing	4 (0.8)	0 (0.0)	
Maternal BMI (%)			0.02
<30	373 (78.5)	62 (67.4)	
≥30	102 (21.5)	30 (32.6)	
Diabetes (%)			0.17
No	421 (88.6)	75 (81.5)	
Gestational diabetes mellitus	34 (7.2)	11 (12.0)	
Diabetes mellitus	20 (4.2)	6 (6.5)	
Smoking during and 3 mo prior to pregnancy (%)			0.11
No	407 (85.7)	72 (78.3)	
Yes	65 (13.7)	20 (21.7)	
Missing	3 (0.6)	0 (0.0)	
Pre-eclampsia (%)			0.14
No	440 (92.6)	83 (90.2)	
Mild	14 (3.0)	3 (3.3)	
Severe	21 (4.4)	5 (5.4)	
Missing	0 (0.0)	1 (1.1)	
HELLP (%)			0.05
No	473 (99.6)	90 (97.8)	
Yes	1 (0.2)	2 (2.2)	
Missing	1 (0.2)	0 (0.0)	
Epilepsy (%)			0.50
No	469 (98.7)	90 (97.8)	
Yes	6 (1.3)	2 (2.2)	
Maternal multivitamin supplement intake			
Preconception[1] (%)			0.32
No	350 (94.3)	69 (97.2)	
Yes	21 (5.7)	2 (2.8)	
First trimester[2] (%)			0.74
≤2 times/wk	43 (10.6)	9 (12.0)	
3–5 times/wk	143 (35.2)	29 (38.7)	
≥5 times/wk	220 (54.2)	37 (49.3)	
Second trimester[3] (%)			0.44
≤2 times/wk	29 (7.1)	8 (10.7)	
3–5 times/wk	147 (35.9)	29 (38.7)	
≥5 times/wk	231 (56.8)	38 (50.7)	
Third trimester[4] (%)			0.42
≤2 times/wk	31 (7.7)	9 (12.2)	
3–5 times/wk	147 (36.4)	27 (36.5)	
≥5 times/wk	226 (55.9)	38 (51.4)	
Child			
Sex (%)			<0.001
Male	192 (40.4)	70 (76.1)	
Female	283 (59.6)	22 (23.9)	
Race-ethnicity (%)			0.62
Black	266 (56.0)	45 (48.9)	
White	32 (6.7)	6 (6.5)	
Hispanic	129 (27.2)	30 (32.6)	
Other	48 (10.1)	11 (12.0)	
Gestational age (%)			0.002
Term	403 (84.8)	66 (71.7)	
Preterm (<37 wk)	72 (15.2)	26 (28.3)	
Year of birth (%)			0.02
1998–2006	152 (32.0)	18 (19.6)	
2007–2014	323 (68.0)	74 (80.4)	

(*Continued*)

TABLE 1 (*Continued*)

Characteristics	Neurotypical ($n = 475$)	ASD ($n = 92$)	*P* value
Sickle cell disease or trait (%)			
SS			0.27
No	469 (98.7)	92 (100.0)	
Yes	6 (1.3)	0 (0.0)	
SC			0.32
No	470 (99.0)	92 (100.0)	
Yes	5 (1.1)	0 (0.0)	
SA			0.07
No	468 (98.5)	88 (95.7)	
Yes	7 (1.5)	4 (4.4)	
Total folate, nmol/L (mean ± SD)	53.6 ± 34.0	58.4 ± 41.8	0.24
5-methyl THF, nmol/L (mean ± SD)	52.4 ± 33.6	57.2 ± 41.2	0.22
UMFA, nmol/L (mean ± SD)	1.24 ± 2.7	1.19 ± 1.1	0.85
DHFR 19-bp deletion (%)[5]			0.79
WT/WT	73 (18.0)	16 (18.8)	
WT/Del	213 (52.5)	47 (55.3)	
Del/Del	120 (29.6)	22 (25.9)	

[1] $n = 442$.
[2] $n = 481$.
[3] $n = 482$.
[4] $n = 478$.
[5] $n = 491$.

Percentage totals may not add-up due to rounding.

Del, deletion; DHFR, dihydrofolatereductase; HELLP, hemolysis, elevated liver enzymes, low platelet count syndrome; SC, hemoglobin SC disease; SA, sickle cell trait (Hb SA); SS, sickle cell disease (Hb SS); THF: tetrahydrofolate; UMFA, unmetabolized folic acid; WT, wild type.

association between higher UMFA and ASD ($aOR_{quartile4}$: 14.70; 95% CI: 2.47, 87.50). When the analysis was repeated to include only preterm children, the OR was statistically significant among those with the highest UMFA (quartile 4) ($aOR_{quartile4}$: 9.69; 95% CI: 1.46, 64.25); however, there was no statistically significant interaction between preterm birth and UMFA ($P > 0.05$).

Next, we assessed the association between cord 5-methyl THF and ASD risk (**Table 4**). Children who had the highest concentrations of cord 5-methyl THF (quartile 4), compared with

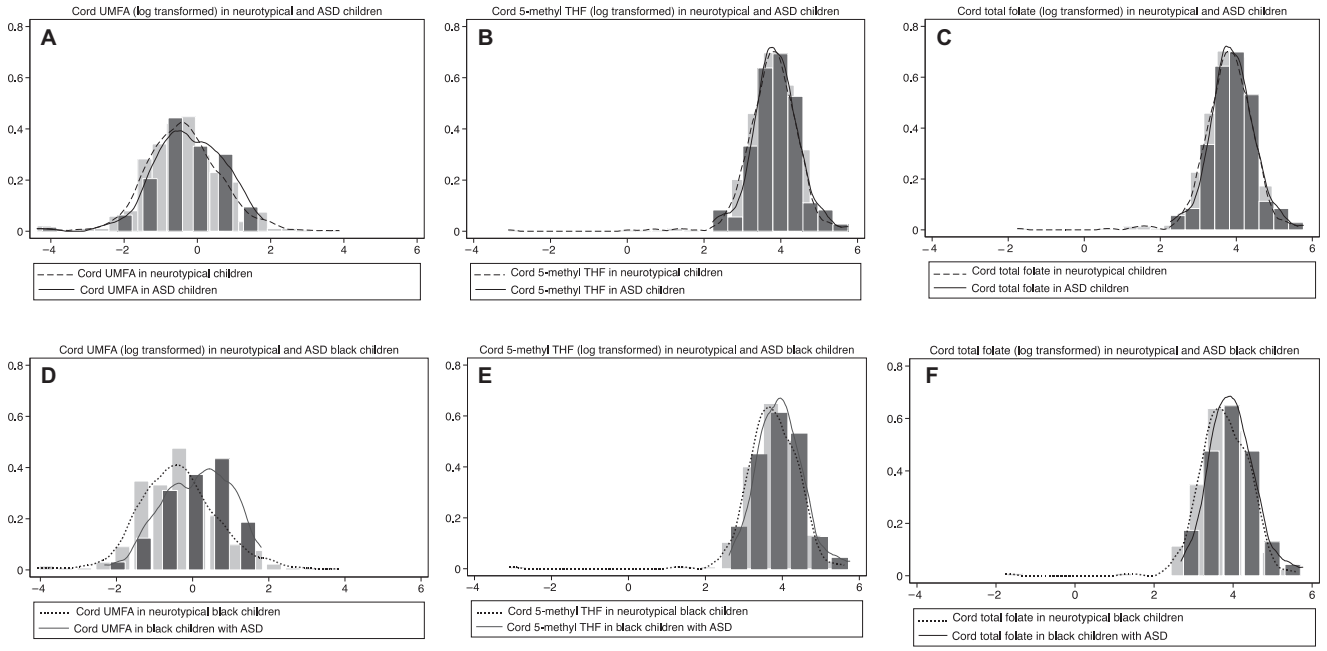

FIGURE 2 Distribution of UMFA, 5-methyl THF, and total folate for neurotypical and ASD children (top row [panels A–C]: overall sample [$n = 567$]; bottom row [panels D–F]: only in blacks [$n = 311$]). ASD, autism spectrum disorder; THF, tetrahydrofolate reductase; UMFA, unmetabolized folic acid.

TABLE 2 Spearman correlation matrix between folate species

Folate species	Cord total folate	Cord 5-methyl THF	Cord UMFA	Maternal blood folate
Cord total folate	1.00			
Cord 5-methyl THF	1.00	1.00		
Cord UMFA	0.39	0.36	1.00	
Maternal blood folate[1]	0.12	0.12	0.16	1.00

[1] $n = 436$.
Cord blood UMFA and 5-methyl THF were measured using the LC-MS/MS method.
Maternal blood folate was measured using chemiluminescent immunoassay.
$P < 0.05$ for all of the correlations.
THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

those with the lowest concentrations (quartile 1), did not have significantly increased ASD risk in unadjusted (OR: 1.63; 95% CI: 0.85, 3.12) or adjusted models (aOR: 1.68; 95% CI: 0.82, 3.44). The lack of significant association was noted even after adjusting for UMFA (model 2) and *DHFR* genotype (model 3). Subgroup analyses to assess the association between cord 5-methyl THF and ASD, by limiting only to black children, showed a significant association between 5-methyl THF (quartile 4) and ASD risk in the unadjusted model (OR: 2.90; 95% CI: 1.06, 7.94). However, this association was no longer significant in the

TABLE 3 Association between log-transformed cord UMFA and ASD in the total sample and in subgroups

UMFA in total sample, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 1	Model 2	Model 3
Q1 (0.25)	142	14	Ref	Ref	Ref	Ref
Q2 (0.51)	142	25	1.95 (0.97, 3.94)	1.76 (0.81, 3.81)	1.73 (0.80, 3.77)	2.05 (0.87, 4.83)
Q3 (0.92)	142	24	1.86 (0.92, 3.76)	1.65 (0.78, 3.53)	1.62 (0.75, 3.47)	1.92 (0.83, 4.45)
Q4 (2.21)	141	29	2.37 (1.19, 4.70)	2.26 (1.08, 4.75)	2.14 (0.98, 4.65)	2.50 (1.05, 5.92)
P-trend			0.02	0.04	0.08	0.05
UMFA in blacks,[1] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 4	Model 5	Model 6
Q1 (0.26)	78	3	Ref	Ref	Ref	Ref
Q2 (0.53)	78	9	3.26 (0.85, 12.54)	5.06 (1.19, 21.56)	4.95 (1.16, 21.19)	6.73 (1.17, 38.77)
Q3 (0.99)	78	13	5.00 (1.36, 18.32)	5.99 (1.46, 24.66)	5.87 (1.42, 24. 24)	9.16 (1.60, 52.29)
Q4 (2.36)	77	20	8.77 (2.48, 30.97)	9.85 (2.53, 38.31)	9.05 (2.20, 37.14)	13.40 (2.34, 76.65)
P-trend			<0.001	0.001	0.002	0.003
UMFA in nonblacks,[2] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 4	Model 5	Model 6
Q1 (0.25)	64	12	Ref	Ref	Ref	Ref
Q2 (0.48)	64	14	1.21 (0.51, 2.88)	0.68 (0.24, 1.98)	0.69 (0.24, 2.02)	0.93 (0.29, 2.94)
Q3 (0.89)	64	9	0.71 (0.28, 1.82)	0.37 (0.13, 1.11)	0.38 (0.13, 1.16)	0.44 (0.14, 1.46)
Q4 (1.82)	64	12	1.00 (0.41, 2.43)	0.85 (0.30, 2.40)	0.87 (0.30, 2.56)	1.13 (0.36, 3.57)
P-trend			0.71	0.52	0.57	0.84
UMFA in PTB,[3] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 7	Model 8	Model 9
Q1 (0.25)	25	3	Ref	Ref	Ref	Ref
Q2 (0.46)	24	8	3.67 (0.84, 16.03)	3.05 (0.47, 19.81)	3.04 (0.47, 19.79)	1.07 (0.12, 9.32)
Q3 (0.91)	25	4	1.40 (0.28, 7.00)	1.11 (0.12, 10.22)	1.03 (0.11, 9.79)	0.79 (0.05, 11.43)
Q4 (2.05)	24	11	6.21 (1.46, 26.43)	9.69 (1.46, 64.25)	8.98 (1.30, 61.88)	5.17 (0.58, 45.84)
P-trend			0.05	0.06	0.09	0.21
UMFA in term blacks, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 10	Model 11	Model 12
Q1 (0.28)	65	3	Ref	Ref	Ref	Ref
Q2 (0.55)	64	5	1.75 (0.40, 7.66)	1.47 (0.31, 7.11)	1.44 (0.29, 7.09)	1.71 (0.28, 10.65)
Q3 (0.97)	64	10	3.83 (1.00, 14.63)	2.63 (0.63, 11.03)	2.60 (0.62, 10.96)	3.80 (0.69, 20.93)
Q4 (2.43)	64	14	5.79 (1.57, 21.26)	3.77 (0.94, 15.13)	3.62 (0.84, 15.59)	5.24 (0.91, 30.03)
P-trend			0.004	0.04	0.05	0.04
UMFA in term nonblacks, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 10	Model 11	Model 12
Q1 (0.25)	53	11	Ref	Ref	Ref	Ref
Q2 (0.46)	53	8	0.68 (0.25, 1.85)	0.61 (0.19, 1.99)	0.57 (0.17, 1.88)	0.67 (0.18, 2.47)
Q3 (0.80)	53	8	0.68 (0.25, 1.85)	0.58 (0.19, 1.76)	0.51 (0.16, 1.64)	0.53 (0.15, 1.92)
Q4 (1.89)	53	7	0.58 (0.21, 1.64)	0.56 (0.17, 1.86)	0.46 (0.12, 1.68)	0.74 (0.19, 2.89)
P-trend			0.33	0.36	0.25	0.60
UMFA in all boys,[4] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 13	Model 14	Model 15
Q1 (0.24)	66	10	Ref	Ref	Ref	Ref
Q2 (0.54)	65	19	2.31 (0.98, 5.46)	1.68 (0.64, 4.39)	1.67 (0.64, 4.38)	2.29 (0.78, 6.78)
Q3 (1.00)	66	19	2.26 (0.96, 5.34)	2.05 (0.81, 5.19)	2.04 (0.80, 5.18)	2.35 (0.82, 6.74)
Q4 (2.30)	65	22	2.87 (1.23, 6.68)	3.10 (1.22, 7.88)	3.05 (1.16, 8.05)	3.40 (1.14, 10.14)
P-trend			0.02	0.02	0.02	0.03

(*Continued*)

TABLE 3 (*Continued*)

UMFA in total sample, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 1	Model 2	Model 3
UMFA in black boys, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 16	Model 17	Model 18
Q1 (0.25)	36	2	Ref	Ref	Ref	Ref
Q2 (0.57)	36	8	4.86 (0.95, 24.75)	6.51 (1.07, 39.59)	6.45 (1.06, 39.42)	16.92 (1.41, 202.92)
Q3 (1.16)	36	11	7.48 (1.52, 36.78)	8.92 (1.52, 52.38)	8.83 (1.49, 52.21)	20.06 (1.64, 245.17)
Q4 (2.88)	36	13	9.61 (1.98, 46.65)	14.70 (2.47, 87.50)	14.27 (2.25, 90.62)	34.92 (2.61, 467.54)
P-trend			0.004	0.003	0.004	0.008

Data are OR, 95% CI in parentheses. Logistic regression analysis was used to estimate the ORs and 95% CIs.

Quartile-specific medians are reported in parentheses.

Model 1: adjusted for maternal age, education, parity, smoking status, diabetes, BMI, race/ethnicity (Black, white, Hispanic, other), child's sex, preterm status, and year of birth.

Model 2: adjusted for model 1 + 5-methyl tetrahydrofolate.

Model 3: adjusted for model 2 + DHFR (total sample size reduced to $n = 491$).

Model 4: adjusted for all covariates in model 1 (except race/ethnicity).

Model 5: adjusted for model 1 (except race/ethnicity) + 5-methyl tetrahydrofolate.

Model 6: adjusted for model 2 (except race/ethnicity) + DHFR (sample size reduced to $n = 261$ [Blacks only analysis] and $n = 230$ [non-Blacks only analysis]).

Model 7: adjusted for all covariates in model 1 (except preterm status).

Model 8: adjusted for model 1 (except preterm status) + 5-methyl tetrahydrofolate.

Model 9: adjusted for model 2 (except preterm status) + DHFR (sample size reduced to $n = 80$).

Model 10: adjusted for all covariates in model 1 (except preterm status and race).

Model 11: adjusted for model 1 (except preterm status and race) + 5-methyl tetrahydrofolate.

Model 12: adjusted for model 2 (except preterm status and race) + DHFR (sample size reduced to $n = 215$ [Blacks-only analysis] and $n = 196$ [non-Blacks–only analysis]).

Model 13: adjusted for all covariates in model 1 (except child's sex).

Model 14: adjusted for model 1 (except child's sex) + 5-methyl tetrahydrofolate.

Model 15: adjusted for model 2 (except child's sex) + DHFR (sample size reduced $n = 228$).

Model 16: adjusted for all covariates in model 1 (except child's sex and race).

Model 17: adjusted for all covariates in model 1 (except child's sex and race) + 5-methyl tetrahydrofolate.

Model 18: adjusted for model 2 (except child's sex and race) + DHFR (sample size reduced to $n = 121$).

[1]The interaction between UMFA (log transformed) and race (non-Blacks versus Blacks) was statistically significant in the crude model ($P = 0.007$) and in model 1 ($P = 0.006$), model 2 ($P = 0.006$), and model 3 ($P = 0.007$).

[2]Non-Blacks included white, Hispanic, and other (including Asian and Pacific Islanders, and individuals with a mixed or other racial background).

[3]The interaction between UMFA (log transformed) and preterm birth (term versus preterm) was not statistically significant in the crude model and models 1, 2, and 3 ($P > 0.05$).

[4]The interaction between UMFA (log transformed) and sex (girls versus boys) was not statistically significant in the crude model and models 1, 2, and 3 ($P > 0.05$).

A Bonferroni correction resulted in a significance level of $P = 0.017$.

ASD, autism spectrum disorder; DHFR, dihydrofolate reductase; PTB, preterm birth; UMFA, unmetabolized folic acid.

adjusted models. There were no significant associations between 5-methyl THF and ASD, when the analysis was restricted to non-Blacks, boys, or preterm infants, and there was no interaction between 5-methyl THF and any of these subgroups (i.e., race, sex, preterm birth) ($P > 0.05$). Similarly, there was no significant association between cord total folate (**Supplementary Table 1**) or *DHFR* genotype and ASD (**Supplementary Table 2**).

We further investigated whether certain covariates influenced UMFA, 5-methyl THF, and total folate concentrations (**Table 5** and **Supplementary Table 3**). Higher cord total folate and second trimester maternal supplement intake were significantly associated with cord UMFA, whereas maternal folate levels was marginally associated with cord UMFA. We also sequentially added covariates to the model and none of the covariates unduly influenced the association between UMFA and ASD (**Supplementary Table 4**). There was a positive association between cord total folate and UMFA, which persisted after stratifying by *DHFR* status and limiting it only to Black children (**Supplementary Figure 3**).

The results of the sensitivity analyses demonstrated consistent association in directionality, with slightly stronger associations noted when the stringent criteria for ASD were applied (**Supplementary Table 5**). Similarly, restricting the analyses to only those children who were diagnosed with ASD after the age of 2 y, yielded a consistent association (data not shown). An additional sensitivity analysis was conducted by excluding participants with *1*) sickle cell disease (SS), hemoglobin SC, or sickle cell trait (SA) (**Supplementary Table 6**), and *2*) Fragile X syndrome (**Supplementary Table 7**) and the findings were overall consistent.

In order to assess whether kidney function influenced the association between UMFA and ASD, we performed additional analyses in a subsample who had cord creatinine data ($n = 283$). Overall, there was a moderate correlation between folate forms and cord creatinine (**Supplementary Table 8**). In the regression analysis, cord creatinine was associated with an increased ASD risk in the crude model; however, the association was no longer statistically significant after adjusting for covariates (**Supplementary Table 9**). Next, we repeated the main analyses in this subsample and noted that the effect sizes were similar before and after adjusting cord creatinine (**Supplementary Table 10**). The association between UMFA and ASD in black children ($n = 167$) was slightly attenuated when the model further adjusted for cord creatinine ($P = 0.05$).

Discussion

Our results show a consistent association between higher cord blood UMFA and increased risk of ASD in black children; in contrast, 5-methyl THF and total folate in cord blood were not associated with an increased ASD risk. To our knowledge, this

TABLE 4 Association between log-transformed cord 5-methyl tetrahydrofolate and ASD in the total sample and in subgroups

5-methyl THF in total sample, nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 1	Model 2	Model 3
Q1 (23.55)	142	18	Ref	Ref	Ref	Ref
Q2 (38.50)	142	24	1.40 (0.72, 2.71)	1.27 (0.62, 2.64)	1.24 (0.60, 2.59)	1.27 (0.59, 2.77)
Q3 (53.60)	142	23	1.33 (0.68, 2.59)	1.28 (0.61, 2.71)	1.24 (0.58, 2.65)	1.37 (0.60, 3.11)
Q4 (85.49)	141	27	1.63 (0.85, 3.12)	1.68 (0.82, 3.44)	1.56 (0.73, 3.34)	1.65 (0.74, 3.68)
P-trend			0.17	0.18	0.28	0.23
5-methyl THF in blacks,[1] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 4	Model 5	Model 6
Q1 (22.95)	78	6	Ref	Ref	Ref	Ref
Q2 (36.81)	78	12	2.18 (0.77, 6.14)	1.74 (0.56, 5.44)	1.66 (0.53, 5.18)	2.53 (0.72, 8.92)
Q3 (53.05)	78	12	2.18 (0.77, 6.14)	2.12 (0.69, 6.50)	1.81 (0.58, 5.63)	2.52 (0.68, 9.38)
Q4 (87.37)	77	15	2.90 (1.06, 7.94)	2.55 (0.85, 7.67)	1.72 (0.54, 5.47)	2.41 (0.66, 8.84)
P-trend			0.05	0.08	0.36	0.21
5-methyl THF in nonblacks,[2] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 4	Model 5	Model 6
Q1 (25.00)	64	13	Ref	Ref	Ref	Ref
Q2 (41.62)	64	11	0.81 (0.33, 1.98)	0.83 (0.30, 2.32)	0.92 (0.32, 2.64)	0.60 (0.19, 1.89)
Q3 (54.06)	64	11	0.81 (0.33, 1.98)	0.83 (0.29, 2.37)	0.99 (0.33, 2.97)	0.92 (0.29, 2.87)
Q4 (85.26)	64	12	0.91 (0.38, 2.17)	0.92 (0.33, 2.56)	1.16 (0.38, 3.51)	0.83 (0.26, 2.66)
P-trend			0.83	0.88	0.77	0.94
5-methyl THF in boys,[3] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 7	Model 8	Model 9
Q1 (23.05)	66	15	Ref	Ref	Ref	Ref
Q2 (37.76)	65	16	1.11 (0.50, 2.49)	0.92 (0.38, 2.24)	0.93 (0.38, 2.27)	0.73 (0.28, 1.87)
Q3 (52.55)	66	19	1.37 (0.63, 3.01)	1.18 (0.49, 2.81)	1.08 (0.45, 2.62)	1.11 (0.42, 2.95)
Q4 (82.05)	65	20	1.51 (0.69, 3.30)	1.34 (0.57, 3.16)	1.13 (0.46, 2.77)	1.17 (0.46, 2.96)
P-trend			0.25	0.41	0.72	0.57
5-methyl THF, in PTB,[4] nmol/L	Total (*n*)	ASD (*n*)	Crude	Model 10	Model 11	Model 12
Q1 (23.64)	25	6	Ref	Ref	Ref	Ref
Q2 (34.63)	24	8	1.58 (0.45, 5.53)	2.81 (0.47, 16.72)	2.59 (0.42, 15.90)	4.41 (0.41, 47.86)
Q3 (50.67)	25	4	0.60 (0.15, 2.47)	0.27 (0.03, 2.31)	0.14 (0.01, 1.76)	0.24 (0.01, 3.90)
Q4 (86.71)	24	8	1.58 (0.45, 5.53)	1.95 (0.32, 11.74)	1.09 (0.16, 7.57)	1.59 (0.17, 14.97)
P-trend			0.84	0.91	0.43	0.70

Data are OR, 95% CI in parentheses. Logistic regression analysis was used to estimate the ORs and 95% CIs.

Quartile-specific medians are reported in parentheses.

Model 1: adjusted for maternal age, maternal education, parity, smoking status, diabetes, BMI, race/ethnicity (Black, white, Hispanic, other), child's sex, preterm status, and year of birth.

Model 2: adjusted for model 1 + UMFA.

Model 3: adjusted for model 2 + DHFR (total sample size reduced to $n = 491$).

Model 4: adjusted for all covariates in model 1 (except race/ethnicity).

Model 5: adjusted for model 1 (except race/ethnicity) + UMFA.

Model 6: adjusted for model 2 (except race/ethnicity) + DHFR.

Model 7: adjusted for all covariates in model 1 (except child's sex).

Model 8: adjusted for model 1 (except child's sex) + UMFA.

Model 9: adjusted for model 2 (except child's sex) + DHFR.

Model 10: adjusted for all covariates in model 1 (except preterm status).

Model 11: adjusted for model 1 (except preterm status) + UMFA.

Model 12: adjusted for model 2 (except preterm status) + DHFR.

[1]The interaction between 5-methyl THF (log transformed) and race (non-Blacks versus Blacks) was not statistically significant in the crude model and models 1, 2, and 3 ($P > 0.05$).

[2]Nonblacks included white, Hispanic, and other (including Asian and Pacific Islanders, and individuals with a mixed or other racial background).

[3]The interaction between 5-methyl THF (log transformed) and sex (girls versus boys) was not statistically significant in the crude model and models 1, 2, and 3 ($P > 0.05$).

[4]The interaction between 5-methyl THF (log transformed) and preterm birth (term versus preterm) was not statistically significant in the crude model and models 1, 2, and 3 ($P > 0.05$).

A Bonferroni correction resulted in a significance level of $P = 0.017$.

DHFR, dihydrofolate reductase; PTB, preterm births; THF, tetrahydrofolate; UMFA, unmetabolized folic acid.

is the first study to examine cord blood folate subtypes and risk of ASD in a prospective US birth cohort. This study extends our previous findings that extremely high maternal plasma folate was associated with ASD (22) and further points to the need to better understand the role of UMFA in ASD.

Consistent with other studies (33–36), UMFA was present in the cord blood of most BBC infants, and as observed in NHANES (36, 37), black children in BBC had higher concentrations than others. The highest cord UMFA was associated with an increased ASD risk in Black children (test of interaction between UMFA and race/ethnicity on ASD was significant). At present, it is unclear why the association between UMFA and ASD was seen only in Black and not in other children. However, we propose several possibilities: first, as reported in a previous NHANES study, non-Hispanic Blacks are twice as likely to have higher UMFA (>1 nmol/L) compared with non-Hispanic whites, Blacks have lower serum and RBC folate concentrations compared with their white counterparts (37). In our study, Black infants had higher UMFA in cord blood compared with non-Black infants. Possible reasons for the difference include genetic or metabolic

TABLE 5 Factors that affect UMFA concentrations (crude and adjusted models)[1]

	Crude		Adjusted	
	β (95% CI)	*P* value	β (95% CI)	*P* value
Maternal age	0.01 (0.00, 0.03)	0.05	0.02 (-0.00, 0.05)	0.08
Education	0.03 (−0.14, 0.21)	0.63	0.15 (−0.18, 0.47)	0.37
Parity	− 0.15 (−0.32, 0.02)	0.09	0.01 (−0.31, 0.34)	0.93
Smoking	0.18 (−0.06, 0.41)	0.15	-0.08 (−0.50, 0.34)	0.70
Diabetes				
No	Ref		Ref	
Gestational diabetes mellitus	0.23 (−0.08, 0.55)	0.14	0.06 (−0.49, 0.62)	0.83
Diabetes mellitus	0.10 (−0.30, 0.51)	0.63	-0.46 (−1.39, 0.47)	0.33
Pre-eclampsia				
No	Ref		Ref	
Mild	0.09 (−0.41, 0.58)	0.73	− 0.24 (−1.08, 0.60)	0.57
Severe	− 0.17 (−0.57, 0.24)	0.42	− 0.33 (1.25, 0.60)	0.49
HELLP				
No	Ref		Ref	
Yes	0.43 (−0.74, 1.59)	0.47	0.37 (-1.18, 1.93)	0.64
BMI	− 0.08 (−0.28, 0.13)	0.47	0.16 (−0.23, 0.54)	0.42
Preterm	− 0.02 (−0.24, 0.21)	0.99	0.04 (−0.44, 0.53)	0.86
Sex	0.10 (−0.07, 0.27)	0.27	0.12 (−0.18, 0.41)	0.43
Race				
Non-Black	Ref		Ref	
Black	0.15 (−0.01, 0.32)	0.07	0.22 (−0.09, 0.52)	0.17
Year of birth (continuous)	− 0.01 (−0.04, 0.01)	0.40	− 0.03 (−0.09, 0.04)	0.43
DHFR[2]				
WT/WT	Ref		Ref	
WT/Del	− 0.14 (−0.38, 0.11)	0.33	− 0.03 (−0.41, 0.36)	0.89
Del/Del	− 0.39 (−0.66, −0.12)	0.006	− 0.17 (−0.62, 0.27)	0.44
Cord blood total folate (log transformed), nmol/L	0.58 (0.46, 0.70)	<0.001	0.71 (0.45, 0.98)	<0.001
Maternal blood folate, nmol/L	0.007 (0.002, 0.01)	0.004	0.01 (0.00, 0.01)	0.05
Maternal multivitamin supplement intake				
First trimester[3]				
≤2 times/wk	Ref		Ref	
3–5 times/wk	0.03 (−0.29, 0.34)	0.85	0.05 (−0.45, 0.54)	0.85
≥5 times/wk	− 0.01 (−0.32, 0.29)	0.93	0.07 (−0.41, 0.54)	0.78
Second trimester[4]				
≤2 times/wk	Ref		Ref	
3–5 times/wk	0.31 (−0.05, 0.67)	0.09	0.63 (0.04, 1.21)	0.04
≥5 times/wk	0.30 (−0.05, 0.65)	0.09	0.67 (0.10, 1.25)	0.02
Third trimester[5]				
≤2 times/wk	Ref		Ref	
3–5 times/wk	0.32 (−0.03, 0.67)	0.08	0.47 (−0.11, 1.05)	0.11
≥5 times/wk	0.32 (−0.02, 0.66)	0.06	0.51 (−0.05, 1.07)	0.08
Creatinine[6]	0.25 (0.12, 0.37)	<0.001	0.09 (−0.07, 0.25)	0.26

Adjusted for all the other covariates.

[1]Total sample size $n = 567$.

[2]DHFR sample size reduced to $n = 491$.

[3]First trimester sample size reduced to $n = 481$.

[4]Second trimester sample size reduced to $n = 482$.

[5]Third trimester sample size reduced to $n = 478$.

[6]Creatinine sample size reduced to $n = 283$.

Del, deletion; DHFR, dihydrofolate reductase; HELLP, hemolysis, elevated liver enzymes, low platelet count syndrome; UMFA, unmetabolized folic acid; WT, wild type.

differences in how folic acid is metabolized (37). Second, preterm birth is a known risk factor for ASD and it is possible that the immature brain of a preterm infant is more vulnerable to environmental insults (38), including elevated UMFA. This is supported by our data that the UMFA and ASD association was stronger in preterm infants than in term infants. Third, males are known to have a higher risk of neurodevelopmental disabilities including ASD (39), ADHD (40), and in this study we found that Black males were particularly at high risk of ASD in the presence of elevated UMFA. Unfortunately, due to our limited sample size, we cannot tease out the independent and synergistic relation of race, preterm birth, sex, and UMFA, which awaits future larger-scale studies. Finally, as this is an observational study, we cannot exclude the possibility that our findings could

be spurious due to unknown confounders; as such, additional studies are definitely needed to confirm our study findings and to delineate the mechanisms.

Research assessing the association between UMFA and neurocognitive outcomes are still emerging; however, at least 1 earlier study showed that UMFA was associated with lower cognitive scores in American seniors; interestingly, 5-methyl THF concentrations were associated with cognitive benefits (41). Although UMFA has not shown to substantially accumulate in the fetus (34), our study suggests that there may be an increased risk even at these low concentrations in Black children. Taken together, these findings raise the possibility that circulating UMFA may have special relevance to the developing brain, especially among male infants and preterm infants.

Maternal folate status is the primary determinant of fetal folate status (42). Although we measured plasma folate status in the mothers, we could not make direct comparisons with the cord total folate because of differences in the assays and time of assessment between maternal and cord blood samples. Also, the circulating UMFA concentration was not measured in the mothers and thus, we could not directly assess the association between maternal and cord UMFA. However, it is plausible that some of the BBC mothers could have had detectable UMFA for the following reasons: BBC is a postfortification birth cohort and nearly all mothers took prenatal supplements (22, 43), both of which are known to result in chronic appearance of UMFA (44). Previous studies have shown that UMFA is ubiquitous in the US population (36, 37) and that the highest UMFA concentrations are observed in those taking supplements and exposed to folic acid fortification (36, 45). In a randomized controlled trial, Pentieva et al. (46) concluded that exposure to folic acid supplements at a dose of 400 μg/d during pregnancy resulted in low or undetectable concentrations of UMFA in mothers and newborns, although cautioned that the effects of higher concentrations of UMFA on metabolic pathways in the fetus are unknown. The majority of pregnant women in the USA consume supplements containing 817 μg/d of folic acid, a dosage much higher than what was studied in the randomized controlled trial (47). Also, based on our earlier study (22), almost a quarter of the mothers had elevated plasma folate around the time of birth (>45.3 nmol/L). NHANES data showed that a higher percentage of UMFA as a percentage of total folate is detected with a serum concentration of >50 nmol/L, when compared with those <50 nmol/L, and the proportional contribution of UMFA is highest in the top decile (~10%) (48, 49).

Folate has a complex relation with ASD development, with studies showing that folate deficiency is associated with an increased risk of ASD and folate supplementation during pregnancy decreases the risk of ASD in the developing offspring (11, 14, 16). On the other hand, our earlier study (22) and a recent Swedish study showed that very high maternal serum folate status during pregnancy may be associated with ASD occurrence in the children (19). To our knowledge, none of the studies have measured the association between UMFA and ASD, thus making it difficult to directly compare our findings with others. The exact mechanism by which the abundance of circulating UMFA may be associated with subsequent ASD risk in Black children is unclear. However, the body of evidence, primarily from animal studies, provides some biological basis for folic acid exposure affecting ASD risk. High maternal folic acid exposure during gestation, in a mouse model, has shown significant alterations in methylation pattern, expression of several genes in the cerebral and cerebellar hemispheres of offspring, and many of these key processes are also impacted in ASD (50–58). In addition, behavioral changes such as increased ultrasonic vocalizations, greater anxiety-like behaviors, short-term memory impairment, hyperactivity, and impairment in reversal learning have been reported in pups exposed to higher folic acid before and during gestation (51, 59, 60).

An alternative hypothesis is that UMFA may not be directly associated with ASD, and could rather merely be a marker of impaired folate export from the kidneys (36, 61). To investigate this, we assessed the association between creatinine, UMFA, and ASD in a subset of BBC children. Although creatinine was moderately correlated with folate species and was associated with ASD in the crude model, it was not significantly associated with ASD risk after accounting for covariates. Additionally, adjusting for creatinine did not substantially alter the association between UMFA and ASD. Taken together, it is uncertain if kidney function indicators could explain the association between UMFA and ASD because our analysis was underpowered due to the limited sample size. Nevertheless, this remains an important question and future studies should further investigate the role of kidney function indicators in the association between UMFA and ASD.

Our study has several limitations that must be considered. First, study children were categorized as neurotypical or ASD based on physician diagnosis as documented in the EMR, and not on research adjudication using Autism Diagnostic Interview-Revised or the Autism Diagnostic Observation Schedule, which would have made the diagnosis more reliable and reduced the chance of potential misclassification. However, this misclassification may not be differential, given the prospective study design and objective lab measurement of UMFA conducted by laboratory personnel who were unaware of the participant's ASD status. As such, any misclassification likely biased the results towards null. Further, restricting to those that had multiple visits indicating an ASD and including diagnosis by a specialist yielded consistent findings. Second, our study was limited to a subset of children who continued to receive pediatric care from the Boston Medical Center. Although there may be concerns about selection bias, this can potentially be assuaged by the fact that the baseline characteristics of our sample and the remaining BBC sample is comparable. Third, we adjusted for well-recognized risk factors, but cannot exclude the possibility of residual confounding due to unknown confounders. Fourth, we did not measure UMFA in maternal circulation and thus cannot attribute the influence of maternal UMFA on cord UMFA. Our ability to compare maternal plasma folate and cord total folate was limited because of differences in the timing of assessment and assay-dependent and interlaboratory differences between maternal and cord folate assessments. Also, our study did not measure the degradation product 4-α-hydroxy-5-methyl tetrahydrofolate; while, if any, might have resulted in random error since it might have not varied based on ASD status. Fifth, we only examined the fetal *DHFR* gene polymorphism and in the future, both maternal and fetal genotypes in *DHFR* and other gene polymorphisms involved in 1-carbon metabolism need to be considered to better understand the environmental and genetic influences on UMFA concentrations and health consequences.

Sixth, the study was conducted in urban, low-income minority children living in the USA with universal mandatory folic acid grain product fortification. The BBC oversampled preterm births, a consistent risk factor for ASD; although this allowed us to study a rare outcome like ASD, it should be noted ASD prevalence in our preterm-enriched cohort was considerably higher than the general population. Therefore, caution should be exercised when extrapolating the findings to other settings, as the findings may not be entirely generalizable. Seventh, additional research is needed to understand the association between other micronutrients in 1-carbon metabolism (e.g. vitamin B-12) and ASD. Finally, the sample size of this study is modest. We were unable to perform more refined subgroup analyses and test for interactions due to limited sample size and power.

In conclusion, in this US urban multiethnic preterm-enriched birth cohort, we found that higher UMFA in cord blood at birth was associated with an increased risk of ASD in Black children. However, there was no association between UMFA in cord blood and ASD in non-Blacks. In contrast, cord blood total folate and 5-methyl THF were not associated with ASD risk in the overall sample and in Blacks and non-Blacks. We would like to emphasize that the findings could be just one piece of the puzzle and should be regarded as hypothesis generating, given the observational nature of the study and the aforementioned limitations. There is no doubt that **optimal** folate status is important for the health of preconception and pregnant women and her developing fetus. The role of folate form, dosage, and duration of intake during pregnancy on the developing fetal brain and child ASD risk is unclear, as is whether this varies by ethnicity, sex, and medical conditions such as prematurity. This is further complicated by maternal and fetal genetic variants relevant to folate metabolism enzymes. We hope that our findings will stimulate additional investigations to further elucidate the UMFA-ASD associations and underlying biological mechanisms. This line of investigation may ultimately help advance precision folate nutrition before and during pregnancy that is tailored to individual mother-fetus' metabolic needs, so as to optimize the health benefits and avoid undue risk (62). Beyond ASD, our previous studies in the BBC have shown that adequate maternal folate may protect against intergenerational cardiometabolic risk (63, 64) and the adverse metabolic effect of prenatal lead exposure (65). As such, what constitutes optimal maternal folate concentrations before and during pregnancy should also consider all the relevant prenatal exposures and a broad array of short- and long-term health outcomes of the mother and her child.

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